# ISOLATION OF EXTENDED SPECTRUM BETA LACTAMASE PRODUCING KLEBSIELLA FROM VARIOUS CLINICAL SAMPLES IN A TERTIARY CARE HOSPITAL

*Dissertation Submitted to*

**THE TAMIL NADU DR. M.G.R. MEDICAL UNIVERSITY**

*in partial fulfillment of the regulations*

*for the award of the degree of*

**M.D. (MICROBIOLOGY)**
**BRANCH – IV**


**GOVT. STANLEY MEDICAL COLLEGE & HOSPITAL**
**THE TAMIL NADU DR. M.G.R. MEDICAL UNIVERSITY**
**CHENNAI, INDIA.**

**MARCH 2008**

# DECLARATION

I solemnly declare that this dissertation "**ISOLATION OF EXTENDED SPECTRUM BETA LACTAMASE PRODUCING KLEBSIELLA FROM VARIOUS CLINICAL SAMPLES IN A TERTIARY CARE HOSPITAL "** is the bonafide work done by me at the Department of Microbiology, Govt. Stanley Medical College and Hospital, Chennai, under the guidance and supervision of **Prof. Dr. P. R. THENMOZHI VALLI, M.D.,** Professor of Microbiology, Govt. Stanley Medical College, Chennai-600 001.

This dissertation is submitted to The Tamil Nadu Dr. M.G.R. Medical University, Chennai in partial fulfillment of the University regulations for the award of degree of M.D. Branch IV Microbiology examinations to be held in March 2008.

Place : Chennai.

Date :

**Dr. J.RAJA**

# CERTIFICATE

This is to certify that this dissertation entitled "**ISOLATION OF EXTENDED SPECTRUM BETA LACTAMASE PRODUCING KLEBSIELLA FROM VARIOUS CLINICAL SAMPLES IN A TERTIARY CARE HOSPITAL "** is the bonafide original work done by **Dr. J. RAJA,** Post graduate in Microbiology, under my overall supervision and guidance in the department of Microbiology, Stanley Medical College, Chennai, in partial fulfillment of the regulations of The Tamil Nadu Dr. M.G.R. Medical University for the award of **M.D Degree in Microbiology (Branch IV).**

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# ACKNOWLEDGEMENT

As the Italian proverb goes,

*It is not enough to aim;*
*You must hit.*

And, here in the following lines, lies my thanks in black and white, for those who helped me ***reach***……

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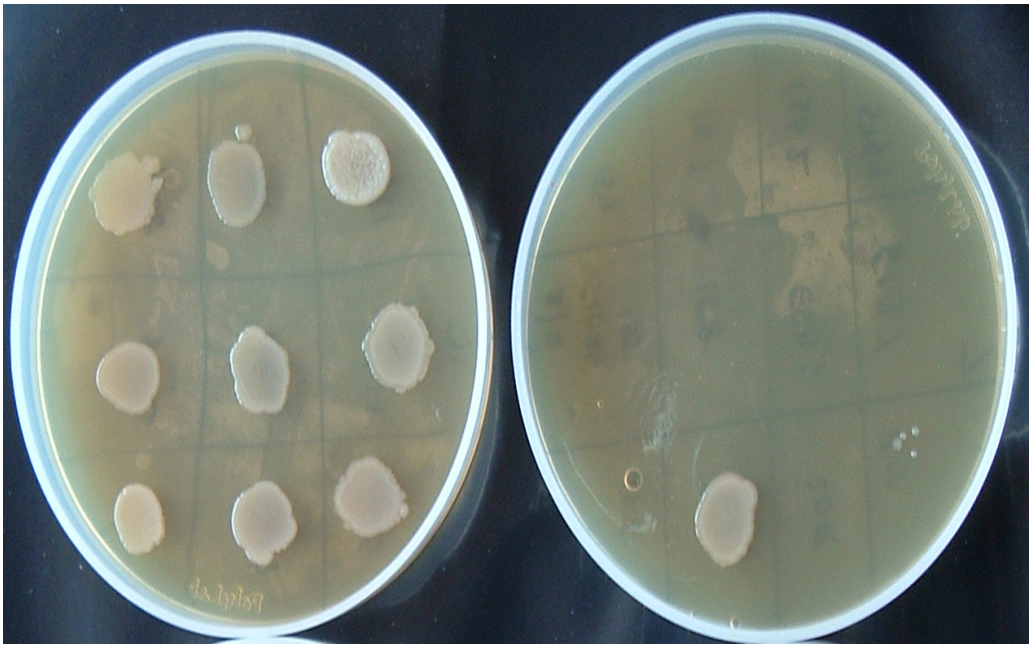
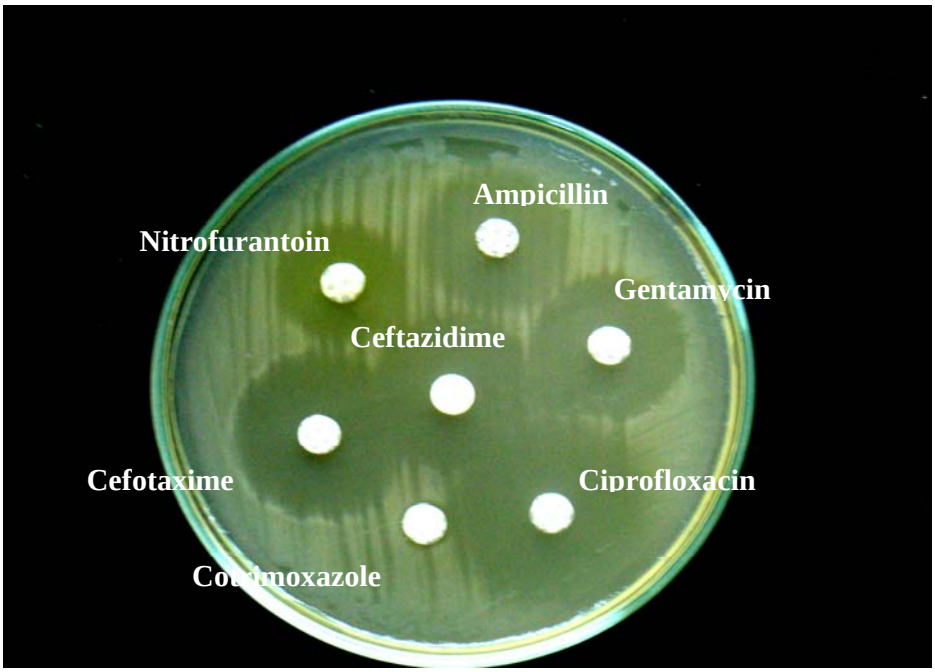
**Minimum Inhibitory Concentration with 4 µg & 1024 µg of Ceftazidime**

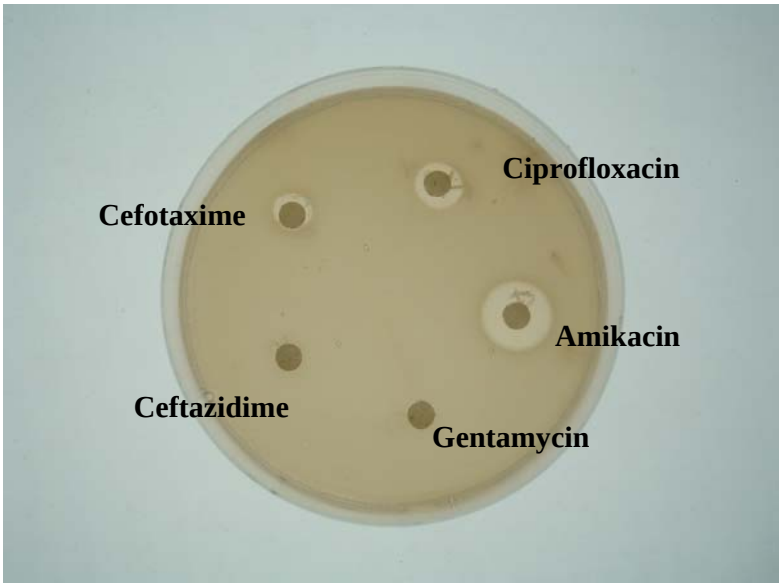


## Non-ESBL Klebsiella antibiogram in Muller Hinton agar plate



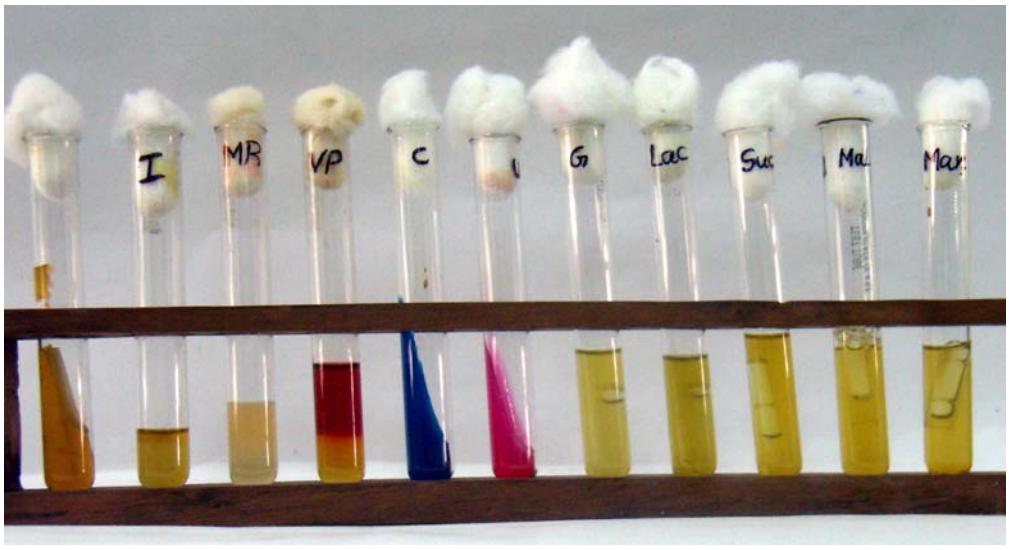

- Organism sensitive to Beta Lactam Antibiotics

## ESBL Klebsiella antibiogram in Muller Hinton agar plate




Organism was resistant to Cefotaxime and Ceftazidime, but has intermediate sensitivity to Amikacin

## Biochemical reactions for the identification of Klebsiella pneumoniae

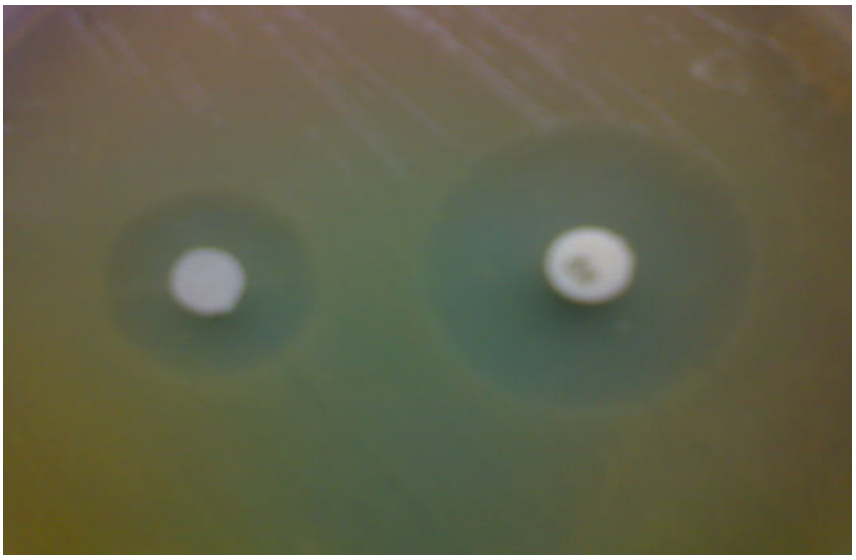



| | | | | | |
|---|---|---|---|---|---|
| **TSI** | **:** | **A/A G+** | **INDOLE** | **:** | **NEGATIVE** |
| **METHY RED** | **:** | **NEGATIVE** | **VOGES PROSKAUER TEST** | **:** | **POSITIVE** |
| **CITRATE** | **:** | **UTILIZED** | **UREASE** | **:** | **POSITIVE** |
| **SUGARS – Glucose, Lactose, Sucrose, Maltose and Mannitol fermented.** | | | | | |

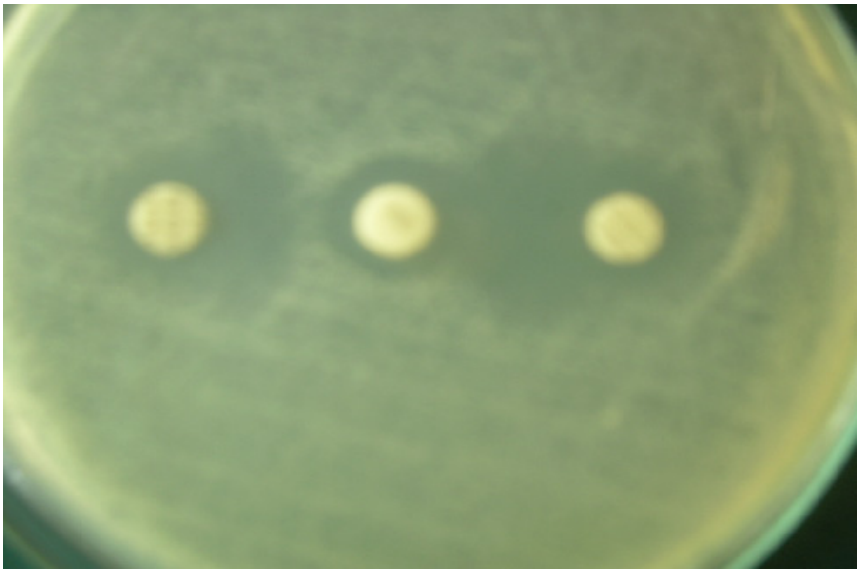
## Biochemical reactions for the identification of Klebsiella oxytoca


| | | | | | |
|---|---|---|---|---|---|
| **TSI** | **:** | **A/A G+** | **INDOLE** | **:** | **POSITIVE** |
| **METHY RED** | **:** | **NEGATIVE** | **VOGES PROSKAUER TEST** | **:** | **POSITIVE** |
| **CITRATE** | **:** | **UTILIZED** | **UREASE** | **:** | **POSITIVE** |
| **SUGARS – Glucose, Lactose, Sucrose, Maltose and Mannitol fermented.** | | | | | |

## PHENOTYPIC CONFIRMATORY METHOD



## DOUBLE DISK SYNERGY TEST



# PCR – THERMOCYCLER


# GEL ELECTROPHORESIS SETUP

## INTRODUCTION

Klebsiella is well known to most clinicians as a cause of community-acquired bacterial pneumonia, occurring particularly in chronic alcoholics, which has a high fatality rate if untreated. The vast majority of Klebsiella infections, however, are associated with hospitalization. As opportunistic pathogens, Klebsiella species primarily attack immunocompromised individuals who are hospitalized and suffer from severe underlying diseases such as diabetes mellitus or chronic pulmonary obstruction. It is estimated that Klebsiella species cause 8% of all nosocomial bacterial infections in the United States and in Europe. No great geographical variations frequency has been noted. In the United States, Klebsiella accounts for 3 to 7% of all nosocomial bacterial infections, placing them among the eight most important infectious pathogens in hospitals, and data collected from the United Kingdom, Italy[22] and from Germany are remarkably similar to those reported by the Centers for Disease Control and Prevention.[50]

The urinary tract is the most common site of infection. Klebsiella accounts for 6 to 17% of all nosocomial urinary tract infections (UTI). As a cause of nosocomial gram-negative

bacteremia, Klebsiella is second only to Escherichia coli. Klebsiella species are often the pathogens involved in neonatal sepsis.

Added to this, due to the extensive spread of antibiotic-resistant strains, especially of extended-spectrum β-lactamase (ESBL) producing strains, there has been renewed interest in Klebsiella infections. Even hospital personnel have elevated rates of Klebsiella carriage. The high rate of nosocomial Klebsiella colonization appears to be associated with the use of antibiotics rather than with factors connected with delivery of care in the hospital.

Since 1982, strains that produce ESBL's, which render them resistant to extended-spectrum Cephalosporins, have evolved. The hallmark of these strains, resistance to ceftazidime, is observed in both Klebsiella pneumoniae and Klebsiella oxytoca isolates. ESBL's are usually plasmid mediated. Since these plasmids are easily transmitted among different members of the Enterobacteriaceae, accumulation of resistance genes results in strains that contain multiresistant plasmids. For this reason, ESBL producing isolates are resistant to a variety of classes of antibiotics. Moreover, the emergence of these multiple resistant Klebsiella strains is unfortunately accompanied by a relatively high

stability of the plasmids encoding ESBL's. Even years after discontinuation of ceftazidime and other extended-spectrum Cephalosporins, continued colonization of patients by ESBL producing Klebsiella strains has been observed. Since ESBL production frequently is accompanied by multiresistance to antibiotics, therapeutic options become limited.

ESBL-producing Klebsiella strains are susceptible to Carbapenems such as Imipenem or Meropenem. Both antibiotics are the drugs of choice in the treatment of infections due to ESBL-producing organisms. In this respect, a recent observation is very disturbing. For the first time, ESBL producing Klebsiella pneumoniae strains, which showed an additional resistance to imipenem, have been isolated. These strains possessed a transmissible plasmid-mediated AmpC-type β-lactamase. [50]

At the same time, these ESBL's are susceptible to drugs of other classes, thereby providing means for timely intervention and successful outcome of the patient. Moreover, in Coimbatore, India 40% of isolates were found to be ESBL producers[8], 68% in Delhi [52] and 87% in Chennai[57]. Indian studies thus gives a higher percentage of ESBL presence compared to international

incidence, which all the more makes this study necessary and imminent for the current scenario.

## AIMS AND OBJECTIVES

- To isolate and speciate Klebsiella obtained from various clinical specimen in the department of Microbiology of Government Stanley Hospital.

- To find out the incidence of ESBL producers amongst Klebsiella species.
- To assess the antibiotic sensitivity pattern of ESBL producing bacteria in comparison with non-ESBL producers.
- To formulate a treatment plan in ESBL harboring patients resistant to routine antibiotics, for effective recovery and for avoiding further emergence and spread of Extended Spectrum β- Lactamases.

## Introduction

The Enterobacteriaceae are a large, heterogeneous group of gram-negative bacilli whose natural habitat is the intestinal tract of humans and animals. So they are also called the coliforms and are the most common group of gram-negative rods cultured in the clinical laboratory and along with staphylococci and streptococci are among the most common bacteria that cause disease. The term “Enterobacteriaceae” was first proposed by Rahn in 1937, and it comprises of organisms that share the following properties. They are gram negative bacilli; do not form spores; are motile by peritrichate flagella or non-motile; grow on peptone or meat extract media without the addition of sodium chloride or other supplements; grow well on MacConkey agar; grow both aerobically and anaerobically; are active biochemically; Presently, 31 genera and 139 species, biogroups and unnamed enteric groups have been defined. The Enterobacteriaceae consists of seven tribes, of which Klebsielleae forms the fifth tribe. The other members of this tribe are the genus Enterobacter, Hafnia, Serratia and Pantoea. [37] Klebsiella species causes various diseases as given in Table below.

**Hospital-acquired bacterial infections caused by *Klebsiella* spp.** [50]

| Infection | % of infections caused by *Klebsiella* | Rank |
|---|---|---|
| UTI | 6–17 | 5–7 |
| Pneumonia | 7–14 | 2–4 |
| Septicemia | 4–15 | 3–8 |
| Wound infections | 2–4 | 6–11 |
| Nosocomial infections | 4–17 | 4–9 |
| Neonatal septicemia | 3–20 | 2–8 |

## History

The genus Klebsiella, initially in 1886 called the "Hyalococcus pneumoniae" [60] was named after Edwin Klebs, a late 19th Century German Microbiologist. The bacillus was also described by Carl Friedländer in 1882, and so was also called as the Friedländer's Bacillus. In the past, the name Klebsiella aerogenes was used for the non-motile, capsulate, gas producing strains commonly found in human faeces and in water. These were similar to strains described in the 19th Century as the "Bakterium lactis aerogenes", referred to later as the "Bacterium aerogenes", and subsequently transferred to the genus Klebsiella. This term Bacterium aerogenes (later Aerobacter aerogenes), was used by water microbiologists to describe a motile strain, presently the

Enterobacter species. To avoid the resultant confusion the species pneumoniae was brought in vogue.[27]

Morphology & Cultural characteristics

Klebsiella are straight, short and thick rods, 0.3 – 1 µm in diameter and 0.6 – 6.0 µm in length, arranged singly, in pairs, or in short chains. Bacilli are capsulated, gram negative, non-motile, facultatively anaerobic chemo-organotropic, having both respiratory and fermentative type of metabolism. [10]

In media rich in carbohydrates, the growth on agar is luxuriant, grayish white and extremely mucoid. Optimal temperature is 37°C (range 12 – 43 °C). In MacConkey agar, they form Pink colonies due to Lactose fermentation. D-Glucose and other carbohydrates are catabolized with the production of acid and gas. There is no haemolysis of horse or sheep red cells. [27]

Epidemiology & Habitat

*Klebsiella* species are ubiquitous in nature. In humans, *K. pneumoniae* is present as a saprophyte in the nasopharynx and in the intestinal tract. The detection rate in stool samples ranges from 5 to 38%, while rates in the nasopharynx range from 1 to 6%. Because gram-negative bacteria do not find good growth conditions on the human skin, *Klebsiella* species are rarely found

there and are regarded simply as transient members of the flora. These carrier rates change drastically in the hospital environment, where colonization rates increase in direct proportion to the length of stay. Even hospital personnel have elevated rates of *Klebsiella* carriage. Reported carrier rates in hospitalized patients are 77% in the stool, 19% in the pharynx, and 42% on the hands of patients. The high rate of nosocomial *Klebsiella* colonization appears to be associated with the use of antibiotics rather than with factors connected with delivery of care in the hospital. Furthermore, widespread use of antimicrobial therapy has often been held responsible for the occurrence of multiple resistant *Klebsiella* strains in hospitals. In the 1970s, the strains were chiefly aminoglycoside-resistant *Klebsiella* strains. Since 1982, strains that produce ESBL's, which render them resistant to extended-spectrum cephalosporins, have evolved. In Europe, the β-lactamases of ceftazidime-resistant *Klebsiella* strains are commonly of the SHV-5 type, whereas TEM-10 and TEM-12 are more prevalent in the United States.[50] In India, the high prevalence of ESBL producing *Klebsiella* species is reported varying from 6 to 87.0 per cent. [3] In Tamilnadu, prevalence varies from 20 to 87 percent. [48] Worldwide the percentage varies from 1.5 to 45 %.[10]

Classification: [50]

## Earlier Classification by:

| Cowan | Bascomb | Ørskov |
|---|---|---|
| *K. aerogenes* | **K. aerogenes / oxytoca** | *K. pneumoniae* |
| *K. edwardsii* | *Edwardsii* | *subsp. pneumoniae* |
| subsp. *edwardsii* | *K. pneumoniae* | subsp. *ozaenae* |
| subsp. *atlantae* | sensu stricto | subsp. *rhinoscleromatis* |
| *K. pneumoniae* | sensu lato | *K. oxytoca* |
| *K. ozaenae* | *K. ozaenae* | *K. terrigena* |
| *K. rhinoscleromatis* | *K. rhinoscleromatis* | *K. planticola (*syn. |
| | *K.* "unnamed group" | *K. trevisanii)* |
| | *Enterobacter aerogenes* | *K. ornithinolytica* |

In 2001, Drancourt and colleagues performed a comparative analysis of the sequences of the 16S rRNA and rpoB genes (encoding the bacterial RNA polymerase β-subunit) of the type strains of nine Klebsiella species, based on which they classified Klebsiella into three clusters[37]

Cluster I: comprises (i) Klebsiella pneumoniae
subspecies (a) pneumoniae,
(b) rhinoscleromatis and
(c) ozaenae
(ii) Klebsiella granulomatis

Cluster II: comprises Klebsiella ornithinolytica,
planticola,
trevisanii and
terrigena

Cluster III: comprises of Klebsiella oxytoca

Later, based on this classification, the genus Klebsiella was divided into two genera, Klebsiella and Raoultella (named after the French bacteriologist Didier Raoult) and Klebsiella oxytoca was left as a monomorphic taxon. Therefore,

Cluster I consists of Klebsiella and

Cluster II consists of Raoutella.

Further, Granier et al have further divided Klebsiella taxon into two clades, corresponding to two genetic groups, called oxy-1 and oxy-2. [37]

Biotyping [37]

| | | Klebsiella oxytoca (%) | Klebsiella pneumoniae (%) | | | Raoultella (Klebsiella) (%) | | |
|---|---|---|---|---|---|---|---|---|
| | | | ozaenae | pneumoniae | rhinoscleromatis | ornithinolytica | planticola | terrigena |
| 1. | Indole Test | 99 | 0 | 0 | 0 | 100 | 20 | 0 |
| 2. | Methyl Red | 20 | 98 | 10 | 100 | 96 | 100 | 60 |
| 3. | Voges Proskauer Test | 95 | 0 | 98 | 0 | 70 | 98 | 100 |
| 4. | Citrate (Simmons) | 95 | 30 | 98 | 0 | 100 | 100 | 40 |
| 5. | Urea Hydrolysis | 90 | 10 | 95 | 0 | 100 | 98 | 0 |
| 6. | Lysine Decarboxylase | 99 | 40 | 98 | 0 | 100 | 100 | 100 |
| 7. | Arginine Dihydrolase | 0 | 6 | 0 | 0 | 0 | 0 | 0 |
| 8. | Ornithine Decarboxylase | 0 | 3 | 0 | 0 | 100 | 0 | 20 |
| 9. | Motility | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 10. | D-Glucose Acid | 100 | 100 | 100 | 100 | 100 | 100 | 100 |
| 11. | D-Glucose Gas | 97 | 50 | 97 | 0 | 100 | 100 | 80 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| 12. | Lactose Fermentation | 100 | 30 | 98 | 0 | 100 | 100 | 100 |
| 13. | Sucrose Fermentation | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 14. | Maltose Fermentation | 100 | 95 | 98 | 100 | 100 | 100 | 100 |
| 15. | D-Mannitol Fermentation | 99 | 100 | 99 | 100 | 100 | 100 | 100 |
| 16. | D-Mannose Fermentation | 100 | 100 | 99 | 100 | 100 | 100 | 100 |
| 17. | Nitrate Nitrite | 100 | 80 | 99 | 100 | 100 | 100 | 100 |
| 18. | Oxidase, Kovac's | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 19. | ONPG | 100 | 80 | 99 | 0 | 100 | 100 | 100 |

Serotyping[50]

It is based mainly on a division according to the capsule antigens. Of 82 capsule antigens described, 77 types form the basis for an internationally recognized capsule antigen scheme. 12 different O-antigen types of *Klebsiella* have also been described.

**Molecular typing methods**[50]

Molecular typing methods, as applied to the genus *Klebsiella*, are still in their infancy. Preliminary descriptions have been presented on plasmid profiles, ribotypes, multilocus enzyme analyses, and applications of pulsed-field gel electrophoresis.

**Pathogenesis**[50]

**(i) Capsular Antigens**

klebsiellae usually develop prominent capsules composed of complex acidic polysaccharides, which can be classified into 77 serological types. Capsules are essential to the virulence of *Klebsiella*. This protects the bacterium from phagocytosis by

polymorphonuclear granulocytes. Strains expressing capsule types K1 and K2 are considered especially likely to be virulent.

**(ii) Pili (Fimbriae)** [50]

Pili (otherwise known as fimbriae) are non-flagellar, filamentous projections on the bacterial surface, helping in attachment to their host. These structures are up to 10 mm long and have a diameter of 1 to 11 nm. There are two predominant types in *Klebsiella* spp.

**(a) Type 1 (common) pili.** They are Mannose sensitive Haemagglutinins (MSHA). These pili bind to mucus or to epithelial cells of the urogenital, respiratory, and intestinal tracts.

**(b) Type 3 pili.** These are mannose-resistant, *Klebsiella*-like hemagglutination (MR/K-HA). These adhere to endothelial cells, epithelia of the respiratory tract, and uroepithelial cells. Three new types of *Klebsiella* adhesins have been recently reported.

(i) The R-plasmid-encoded CF29K adhesin of *Klebsiella pneumoniae* mediate adherence to the human intestinal cell lines.

(ii) Another new *Klebsiella* adhesin characterized by aggregative adhesion to intestinal cell lines

The two adhesins mentioned above are non-fimbrial.

(iii) A new fimbria KPF-28 is putative colonization factor of the human gut. Interestingly, this fimbrial type has been found in the

majority of *Klebsiella pneumoniae* strains producing CAZ-5/SHV-4 type ESBL.

**(iii) Siderophores**[50]

Iron is an essential factor in bacterial growth, functioning mainly as a redox catalyst in proteins participating in oxygen and electron transport processes. Under iron-deficient conditions, e.g., in the host milieu, enterobacteria synthesize a variety of siderophores, which belong to two different chemical groups, one consisting of the phenolate-type siderophores siderophores and one consisting of the hydroxamate-type siderophores.

**(iv) Lipopolysaccharides**[50]

Due to their endotoxic properties, LPS are considered important in the pathology of septicemia. The small number of different *Klebsiella* O-types is a great advantage with respect to their applicability as vaccines. Only eight O types are known, O1 being the most commonly found O type in clinical isolates.

**(v) Capsular Polysaccharides**[50]

CPS has been the obvious vaccine candidates for several reasons. Capsules are produced by almost all *Klebsiella* strains; they represent the outermost layer of surface structures in contact with the host milieu, and they have been proven to be highly immunogenic and nontoxic.

## Antibiotic Sensitivity[50]

**Inherently, klebsiella is sensitive to certain drugs, like β-lactam antibiotics, sulphonamides, fluoroquinolones, aminoglycosides, chloramphenicol and nitrofurantoin. They are likewise resistant by their genetic makeup to erythromycin, roxithromycin, rifampicin, fusidic acid, lincosamides, glycopeptides and streptogramins.**

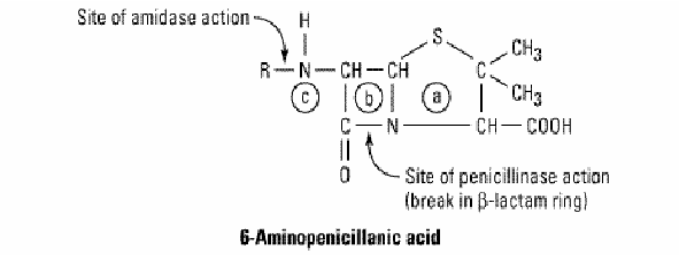
Natural Sensitivity pattern of klebsiella[50]

| | Benzylpenicillin, oxacillin | Amoxicillin | Mezlocillin, ampicillin/sulbactam | Other penicillins | All tested cephalosporins | All tested carbapenems | Aztreonam | Erythromycin, Azithromycin | All tested tetracyclines | Sulphamethoxazole | Trimethoprim, co- | All tested quinolones | All tested | Chloramphenicol | Nitrofurantoin |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Klebsiella pneumoniae** | R | R | S-I | s | s | s | s | R | S-I | R ?t | s | s | s | s | s |
| **Klebsiella ozaenae** | R | I-R | s | s | s | s | s | R | S-I | s | s | s | s | s | s |
| **Klebsiella rhinoscleromatis** | R | R | s | s | s | s | s | I | s | s | s | s | s | s | s |
| ***Klebsiella oxytoca*** | R | R | S-I | s | s | s | s | R | S-I | s | s | s | s | s | s |
| ***Klebsiella planticola*** | R | R | s | s | s | s | s | R | S-I | s | s | s | s | s | s |
| ***K. terrigena*** | R | I-R | s | s | s | s | s | R | S-I | s | s | s | s | s | s |

## Beta Lactam antibiotics:[11]

These are antibiotics having a beta-lactam ring[26], consisting of

i) Penicillins
ii) Cephalosporins
iii) Monobactams
iv) Carbapenems




**6-Aminopenicillanic acid**

The following structures can each be substituted at the R to produce a new penicillin.

## Mechanism of action of Beta Lactams

ß-lactam antibiotics, inhibit bacterial growth by interfering with a specific step in bacterial cell wall synthesis. The cell wall is composed of a complex crosslinked polymer, peptidoglycan consisting of polysaccharides and polypeptides. The polysaccharide contains alternating amino sugars, *N*-acetylglucosamine and *N*-acetylmuramic acid. A five-amino-acid peptide is linked to the *N*-acetylmuramic acid sugar. This peptide terminates in D-alanyl-D-alanine. Penicillin-binding proteins (PBPs) catalyze the transpeptidase reaction that removes the terminal alanine to form a crosslink with a nearby peptide, which gives cell wall its structural rigidity. Beta-Lactam antibiotics are structural analogs of the natural D-Ala-D-Ala substrate and they are covalently bound by PBPs at the active site. After a Beta–lactam antibiotic has attached

to the PBP, the transpeptidation reaction is inhibited, peptidoglycan synthesis is blocked, and the bacterium dies.

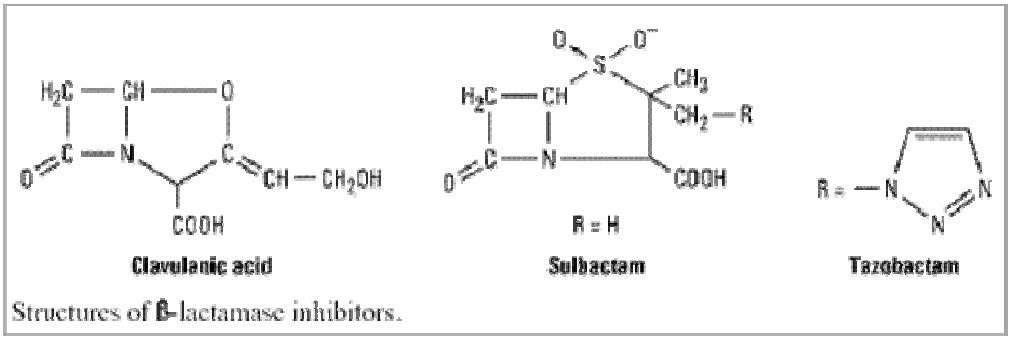
## Cephalosporins

The nucleus of the cephalosporins, 7-aminocephalosporanic acid, bears a close resemblance to 6-aminopenicillanic acid. The intrinsic antimicrobial activity of natural cephalosporins is low, but the attachment of various R1 and R2 groups has yielded drugs of good therapeutic activity and low toxicity. First-generation compounds have better activity against gram-positive organisms and the later compounds exhibit improved activity against gram-negative aerobic organisms.

## Monobactams

These are drugs with a monocyclic β-lactam ring. They are relatively resistant to β-lactamases and active against gram-negative bacilli. They have no activity against gram-positive bacteria or anaerobes. Aztreonam is the only monobactam available. It resembles aminoglycosides in its spectrum of activity.

## Beta Lactamase Inhibitors[35]



Structures of β-lactamase inhibitors.

- Clavulanic acid
- Sulbactam
- Tazobactam

These substances resemble β-lactam molecules but by themselves have very weak antibacterial action. They are potent inhibitors of many but not all bacterial lactamases and can protect hydrolyzable penicillins from inactivation by these enzymes. β-Lactamase inhibitors are most active against Ambler class-A β-lactamases (plasmid-encoded transposable element [TEM] β -lactamases in particular) such as those produced by staphylococci, H influenzae, N gonorrhoeae, salmonella, shigella, E coli, and K pneumoniae. They are not good inhibitors of class C β-lactamases, which typically are chromosomally encoded and inducible, produced by enterobacter, citrobacter, serratia, and pseudomonas, but they do inhibit chromosomal β-lactamases of legionella, bacteroides, and branhamella.

## Carbapenems

The carbapenems are structurally related to β-lactam antibiotics. They are indicated in infections due to organisms resistant to other drugs.[33] Ertapenem, imipenem, and meropenem are licensed for use in the USA. Imipenem has a wide spectrum with good activity against many gram negative bacilli, including *Pseudomonas aeruginosa,* gram-positive organisms, and anaerobes. It is resistant to most β-lactamases but not metallo β-lactamases. *Enterococcus faecium,* methicillin-resistant strains of staphylococci, *Clostridium difficile, Burkholderia cepacia,* and *Stenotrophomonas maltophilia* are resistant. Imipenem is inactivated by dehydropeptidases in renal tubules, resulting in low urinary concentrations. Consequently, it is administered together with an inhibitor of renal dehydropeptidase, cilastatin, for clinical use. Meropenem is similar to imipenem but has slightly greater activity against gram-negative aerobes and slightly less activity against gram-positives. It is not significantly degraded by renal dehydropeptidase and does not require an inhibitor. Ertapenem is less active than meropenem or imipenem against P*seudomonas aeruginosa* and Acinetobacter species. It is not degraded by renal dehydropeptidase.

## Extended Spectrum Beta Lactamases (ESBL)-

**Definition:** ESBLs are β-lactamases capable of conferring bacterial resistance to the penicillins, first-, second-, and third-generation cephalosporins, and aztreonam (but not the cephamycins or carbapenems) by hydrolysis of these antibiotics, and which are inhibited by β-lactamase inhibitors such as clavulanic acid. [18]

### Evolution of ESBLs[19]

Emergence of resistance to β-lactam antibiotics began even before the first β-lactam penicillin was developed. The first β-lactamase was identified in *Escherichia coli* prior to the release of penicillin for use in medical practice. The first plasmid-mediated β-lactamase in gram-negatives, TEM-1, was described in the early 1960s. The TEM-1 enzyme was originally found in a single strain of *E. coli* isolated from a blood culture from a patient named Temoniera in Greece, hence the designation TEM. Likewise, another common plasmid mediated β-lactamase found in *Klebsiella pneumoniae* and *E.coli* is SHV-1 (for sulphydryl variable). The SHV-1 β-lactamase is chromosomally encoded in the majority of isolates of *K. pneumoniae* but is usually plasmid mediated in *E. coli*. Over the last 20 years, many new β-lactam antibiotics have been developed

that were specifically designed to be resistant to the hydrolytic action of β-lactamases. However, with each new class that has been used to treat patients, new β-lactamases emerged that caused resistance to that class of drug. Not surprisingly, resistance to these expanded-spectrum β-lactam antibiotics due to β-lactamases emerged quickly. This was due to continuous mutation of the enzymes. [25] The first of these enzymes capable of hydrolyzing the newer β-lactams, SHV-2, was found in a single strain of *Klebsiella ozaenae* isolated in Germany. Because of their increased spectrum of activity, especially against the oxyimino-cephalosporins, these enzymes were called extended-spectrum β-lactamases (ESBLs). Today, over 575[61] different ESBLs have been described.

**Classification of ESBLs:**

Various classification schemes have been proposed by many researchers.

i) **Classification of Sawai *et al*** in 1968 was based on response to antisera.

ii) **Richmond and Sykes scheme** in 1973 was on the basis of substrate profile.

iii) Extension of the Richmond and Sykes scheme by **Sykes and Mathew** in 1976 was based on differentiation by isoelectric focussing.

iv) In the scheme proposed by **Mitsuhashi and Inoue** in 1981, the category “cefuroxime hydrolyzing β-lactamases” was added to “penicillinase and cephalosporinase” classification.

v) The groupings proposed by **Bush** in 1989, was based on correlation of substrate and inhibitory properties with molecular structure.

However, the number and variety of enzymes have proliferated beyond the boundaries of the scheme.

vi) A more modern scheme based on molecular structure classification was proposed by **Ambler** includes, of necessity, only those enzymes that have been characterized.

vii) Recently a new classification scheme has been developed to integrate functional and molecular characteristics. The **Bush-Jacoby-Medeiros scheme**[15] puts 178 β-lactamases from naturally occurring bacterial isolates into four groups based on substrate and inhibitor profiles.

*Classification schemes for bacterial b-lactamases*[15, 36]

| Jacoby-Medeiros | 1989 Bush group | Richmond-Sykes class | Mitsuhashi-Inoue type | Molecular class | Preferred substrates | Inhibited by CA EDTA | | Representative enzymes |
|---|---|---|---|---|---|---|---|---|
| 1 | 1 | Ia, Ib, Id | CSase | C | Cephalosporins | - | - | AmpC enzymes from gram-negative bacteria; MIR-1 |
| 2a | 2a | Not included | PCase V | A | Penicillins | + | - | Penicillinases from gram-positive bacteria |
| 2b | 2b | III | PCase I | A | Penicillins, cephalosporins | + | - | TEM-1, TEM-2, SHV-1 |
| 2be | 2b' | Not included except K1 in class IV | CXase | A | Penicillins, narrow- & extended-spectrum cephalosporins, monobactams | + | - | TEM-3 to TEM-26, SHV-2 to SHV-6, Klebsiella oxytoca K1 |
| 2br | Not included | Not included | Not included | A | Penicillins | +/- | - | TEM-30 to TEM-36, TRC-1 |
| 2c | 2c | II, V | PCase IV | A | Penicillins, carbenicillin | + | - | PSE-1, PSE-3, PSE-4 |
| 2d | 2d | V | PCase II, PCase III | D | Penicillins, cloxacillin | +/- | - | OXA-1 to OXA-11, PSE-2 (OXA-10) |
| 2e | 2e | Ic | CXase | A | Cephalosporins | + | - | Inducible cephalosporinases from Proteus vulgaris |
| 2f | Not included | Not included | Not included | A | Penicillins, cephalosporins, carbapenems | + | - | NMC-A from Enterobacter cloacae, Sme-1 from Serratia marcescens |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| 3 | 3 | Not included | Not included | B | Most b-lactams, including carbapenems | - | + | L1 from Xanthomonas maltophilia, CcrA from Bac-teroides fragilis |
| 4 | 4 | Not included | Not included | ND[c] | Penicillins | - | ? | Penicillinase from Pseudomonas cepacia |

*a* Csase, cephalosporinase; PCase, penicillinase; CXase, cefuroxime-hydrolyzing b-lactamase.
*b* CA, clavulanic acid.
*c* ND, not determined.

**Detection of ESBLs:** [18]

**Clinical Laboratory Standards Institute (CLSI) Recommended Methods for ESBL Detection:**

**I. Screening for ESBL producers-**

**(i) Disk diffusion method:**

The CLSI has proposed disk diffusion methods for screening for ESBL production by Klebsiellae, *Escherichia coli*, and *Proteus mirabilis*. Laboratories using disk diffusion methods for antibiotic susceptibility testing can screen for ESBL production by noting specific zone diameters, which indicate a high level of suspicion for ESBL production. Cefpodoxime, ceftazidime, aztreonam, cefotaxime, or ceftriaxone is used. However, the use of more than one of these agents for screening improves the sensitivity of detection. If any of the zone diameters indicate suspicion for ESBL

production, phenotypic confirmatory tests should be used to ascertain the diagnosis.

## II. Phenotypic Confirmatory Tests for ESBL Production

**(i) Cephalosporin/clavulanate combination disks.**

The CLSI advocates use of cefotaxime (30 µg) or ceftazidime disks (30 µg) with or without clavulanate (10 µg) for phenotypic confirmation of the presence of ESBLs in klebsiellae and *Escherichia coli*. The disk tests are to be performed with confluent growth on Mueller- Hinton agar. A difference of 5 mm between the zone diameters of either of the cephalosporin disks and their respective cephalosporin/clavulanate disk is taken to be phenotypic confirmation of ESBL production.

**(ii) Broth microdilution.** Phenotypic confirmatory testing can also be performed by broth microdilution assays using ceftazidime (0.25 to 128 µg/ml), ceftazidime plus clavulanic acid (0.25/4 to 128/4 µg/ml), cefotaxime (0.25 to 64 µg /ml), and cefotaxime plus clavulanic acid (0.25/4 to 64/4 µg/ml). Again it should be emphasized that both ceftazidime and cefotaxime should be used. A 3-twofold-serial-dilution decrease in MIC of either cephalosporin in the presence of clavulanic acid is compared to its MIC when tested alone.

**Quality control when performing screening and phenotypic confirmatory tests:** Non-ESBL-producing organism (*Escherichia coli* ATCC 25922) and an ESBL-producing organism (*Klebsiella pneumoniae* ATCC 700603) are used as controls.

**Implications of positive phenotypic confirmatory tests:** According to CLSI guidelines, isolates which have a positive phenotypic confirmatory test should be reported as resistant to all cephalosporins (except the cephamycins, cefoxitin, and cefotetan) and aztreonam, regardless of the MIC of that particular cephalosporin.

**III. Commercially Available Methods for ESBL Detection**

**(i) Etest for ESBLs.** AB Biodisk (Solna, Sweden) produces plastic drug-impregnated strips, one end of which contains a gradient of ceftazidime (MIC test range 0.5 to 32 µg/ml) and the other with a gradient of ceftazidime plus a constant concentration of clavulanate (4 µg /ml). The reported sensitivity of the method as a phenotypic confirmatory test for ESBLs is 87 to 100% and the specificity is 95 to 100%. The manufacturer currently recommends a 8-fold reduction in cephalosporin MICs in the presence of clavulanate.

**(ii) Vitek ESBL cards.** Vitek ESBL test (bioMerieux Vitek, Hazelton, Missouri) utilizes cefotaxime and ceftazidime, alone (at

0.5 µg/ml), and in combination with clavulanic acid (4 µg/ml). Inoculation of the cards is identical to that performed for regular Vitek cards. Analysis of all wells is performed automatically once the growth control well has reached a set threshold (4 to 15 hours of incubation). A predetermined reduction in the growth of the cefotaxime or ceftazidime wells containing clavulanic acid, compared with the level of growth in the well with the cephalosporin alone, indicates a positive result. Sensitivity and specificity of the method exceed 90%.

**(iii) MicroScan panels.** Dade Behring MicroScan (Sacramento, Calif.) produces dehydrated panels for microdilution antibiotic susceptibility testing.

**(iv) BD Phoenix Automated Microbiology System.** The Phoenix ESBL test uses growth response to cefpodoxime, ceftazidime, ceftriaxone and cefotaxime, with or without clavulanic acid, to detect the production of ESBLs. Results are usually available within 6 hours.

In a study,[4] the test sensitivities for MicroScan ESBL plus ESBL confirmation panel was 100%, VITEK 1 GNS-120 99%, Etest ESBL 97% and BD BBL Sensi-Disk ESBL Confirmatory Test disks 96%.

## IV. Other Methods for ESBL Detection

**(i) Cephalosporin/clavulanate combination disks on Iso-Sensitest agar.** The British Society for Antimicrobial Chemotherapy has recommended the disk-diffusion method for phenotypic confirmation of ESBL presence using ceftazidime/ clavulanate and cefotaxime/clavulanate combination disks, although recommends that the test be performed with semiconfluent growth on Iso-Sensitest agar (rather than confluent growth on Mueller-Hinton agar).

**(ii) Double-disk diffusion test.** In the late 1980s, French investigators[13,32] described a disk diffusion test in which synergy between cefotaxime and clavulanate was detected by placing a disk of amoxicillin/clavulanate (20 µg/10 µg) and a disk of cefotaxime (30 µg), 30 mm apart (center to center) on an inoculated agar plate. A clear extension of the edge of the cefotaxime inhibition zone toward the disk containing clavulanate was interpreted as synergy, indicating the presence of an ESBL;

**(iii) Agar supplemented with clavulanate.** Ho et al. [31] described a method by which Mueller-Hinton agar was supplemented with 4 µg/ml of clavulanate. Antibiotic disks containing ceftazidime (30 µg), cefotaxime (30 µg), ceftriaxone (30 µg), and aztreonam (30 µg) were placed on the clavulanate-containing agar and on regular

clavulanate free Mueller-Hinton agar plates. A difference in β-lactam zone width of 10 mm on the two media was considered positive for ESBL production.

**(iv) Disk replacement method.** Casals and Pringler (Mediterranean Congress of Chemotherapy, Barcelona, Spain, 20 to 25 May 1990) described the following method. Three amoxicillin/clavulanate disks are applied to a Mueller-Hinton plate inoculated with the test organism. After 1 hour at room temperature, these antibiotic disks are removed and replaced on the same spot by disks containing cefotaxime, ceftazidime and aztreonam. Control disks of these three antibiotics are simultaneously placed at least 30 mm from these locations. A positive test is indicated by a zone increase of 5 mm for the disks which have replaced the amoxicillin/clavulanate disks compared to the control disks.

**(v) Three-dimensional test.** The three-dimensional test gives phenotypic evidence of ESBL-induced inactivation of extended-spectrum cephalosporins or aztreonam without relying on demonstration of inactivation of the β-lactamases by a β-lactamase inhibitor. In this test, developed by Thomson, the surface of the susceptibility plate is inoculated by standard methods for disk diffusion testing, but additionally a circular slit is cut in the agar

concentric with the margin of the plate. A heavy inoculum of the test organism ($10^9$ to $10^{10}$ CFU of cells) is pipetted into the slit. β-Lactam-impregnated disks are then placed on the surface of the agar 3 mm outside of the inoculated circular slit. β-Lactamase-induced inactivation of each test antibiotic is detected by inspection of the margin of the zone of inhibition in the vicinity of its intersection with the circular three-dimensional inoculation. The presence of β-lactamase-induced drug inactivation is visualized as a distortion or discontinuity in the usually circular inhibition zone or the production of discrete colonies in the vicinity of the inoculated slit.

**(vi) Molecular ESBL detection techniques**[48]

| Test | Advantages | Disadvantages |
|---|---|---|
| DNA probes | Specific for gene family (e.g., TEM or | Labor intensive, cannot distinguish between ESBLs and |

| | | |
|---|---|---|
| | SHV) | non-ESBLs, cannot distinguish between variants of TEM or SHV |
| PCR | Easy to perform, specific for gene family (e.g.,TEM or SHV) | Cannot distinguish between ESBLs and non-ESBLs, cannot distinguish between variants of TEM or SHV |
| Oligotyping | Detects specific TEM variants | Requires specific oligonucleotide probes, labor intensive, cannot detect new variants |
| PCR-RFLP | Easy to perform, can detect specific nucleotide changes | Nucleotide changes must result in altered restriction site for detection |
| PCR-SSCP | Can distinguish between a number of SHV variants | Requires special electrophoresis conditions |
| LCR | Can distinguish between a number of SHV variants | Requires a large number of oligonucleotide primers |
| Nucleotide sequencing | The gold standard, can detect all variants | Labor intensive, can be technically challenging, can be difficult to interpret manual methods |

## TREATMENT AND OUTCOME OF INFECTIONS WITH ESBL-PRODUCING ORGANISMS

### In Vitro Studies

Given the ability of ESBL-producing organisms to hydrolyze many β-lactam antibiotics, it is not surprising that antibiotic choice for infections with such organisms is seriously reduced.

### Antibiotic Choice for Serious Infections:

## Recommended Treatment for Infections with ESBL producers[18]

| S.No | Infection Type | Therapy of choice | Second-Line Therapy |
|---|---|---|---|
| 1. | Urinary tract Infection | Quinolone* | Amoxycillin/Clavulanate |
| 2. | Bacteremia | Carbapenem | Quinolone* |
| 3. | Hospital acquired pneumonia | Carbapenem | Quinolone* |
| 4. | Intra-abdominal infection | Carbapenem | Quinolone*(plus Metronidazole) |
| 5. | Meningitis | Meropenem | Intrathecal Polymyxin B |
| * If the organism is susceptible | | | |

Third-generation cephalosporins are poor choices for the treatment of serious infections due to ESBL producing organisms. Failure rates are high when MICs of the cephalosporins are elevated (for example, 4 or 8 µg/ml) but still within the susceptible range. Cefepime should not be used as first-line therapy against ESBL-producing organisms; if it is used (for example, against organisms with a cefepime MIC of 2 µg/ ml), it should be used in high dosage (at least 2 g twice a day). In vitro synergy may be achievable between cefepime and amikacin.

Carbapenems should be regarded as the drugs of choice for serious infections with ESBL-producing organisms. In nosocomial meningitis, meropenem should be regarded as the drug of choice.

## MATERIALS & METHODS

The present bacteriological study was carried out on samples collected from patients admitted in the hospital as Inpatients and also patients attending the Outpatient Department of Government Stanley Hospital, from June, 2006 to May, 2007.

A total of 204 klebsiella were isolated from 1700 specimen collected and processed in the Department of Microbiology, Stanley Medical College, Chennai and were included in this study.

**Criteria for selection of ESBL producing Klebsiella:** Routine disk diffusion susceptibility testing was performed by modified Kirby bauer method following the criteria put forward by the CLSI, with 30 µg of 3$^{rd}$ generation Cephalosporins. Isolates found resistant to these drugs were selected for the presence of ESBL.

**Materials :**

Isolates obtained from various clinical samples like urine, pus, sputum and blood were analysed for ESBL production.

**Methods:**

The methodology includes –

1. Collection of specimen
2. Specimen Processing
3. Identification of the pathogen
4. Antimicrobial sensitivity testing
5. Initial Screening test
6. Phenotypic Confirmatory test
7. Double disk diffusion test (DDST)
8. Minimum Inhibitory Concentration (MIC) Determination
9. CLSI 2006 Protocol
10. Polymerase Chain Reaction (PCR) for detection of ESBL producing genes

1. **Collection of specimen:**

**Urine:** Urine samples were collected, by sampling the midstream flow, following the Clean-Catch technique. The

purpose was to collect the urine that has been sitting in the bladder, discarding the initial portion that has been in contact with the urethra and presumably contaminated with urethral flora.[37] In paedriatic patients, urine flow was stimulated by tapping just above the pubis with two fingers one hour after the feed; one tap/second was given for one minute, an interval of one minute was allowed, then tapping was resumed in this cycle.[41]

In case of an indwelling catheter, the catheter collection port was disinfected with 70% ethanol, and 5 to 10 ml of urine was aspirated with needle and syringe.

The collected specimen was transported to the lab within 2 hours, and in case of delay in processing, was kept at 4˚C for a maximum of 24 hours.[9]

**Pus:** The wound area was wiped with sterile saline, and the swab was rolled along the leading edge of the wound. The collected specimen was transported to the lab within 2 hours, and in case of delay in processing, were kept at 4˚C for a maximum of 24 hours.[9]

**Body fluids:** The skin was disinfected before aspirating the sample, and needle aspiration was done. The sample was transported at room temperature and processed without delay.

**Sputum:** The patient was advised to rinse or gargle with water before collection, and specimen collected from a deep cough.

**Blood:** Sampling of blood was done before starting antibiotics, and during febrile episodes. If the patient was already on antibiotics, then blood was drawn just before the next dose of drug. The venipuncture site was cleaned with 1% iodine and allowed to dry for 1 – 2 minutes and then the area was wiped with a 70% alcohol wash and allowed to dry. Blood was drawn upto 10 ml in adults and 5 ml in children, aseptically using a syringe and needle. Without removing the needle, blood was transferred to a Brain-Heart Infusion broth containing bottle containing 50 ml of media, giving an overall dilution of 1:5 to 1:10. The bottle was incubated at 37° C.[41]

**2. Specimen Processing:**

Once the specimen was received in the laboratory, it was cultured onto Blood agar plate and MacConkey agar plate and incubated aerobically at 37˚ C for 18 to 24 hours.

**3. Identification of the pathogen:**

Non-motile Short stout gram negative bacilli on gram staining, and large, non-hemolytic, greyish white mucoid colonies on Blood agar plate and pink colored lactose fermenting mucoid colonies in MacConkey agar plate suggests Klebsiella species.

Biochemical reactions for their identification were as follows:

| Organism | TSI | Indole | MR | VP | Citrate | Oxidase | Catalase | Sugar Fermentation | | | | | Motility |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | Glucose | Lactose | Sucrose | Maltose | Mannitol | |
| Klebsiella Pneumonia | A/A G+ | N | N | P | Utilized | N | P | P | P | P | P | P | N |
| Klebsiella oxytoca | A/A G+ | P | N | P | Utilized | N | P | P | P | P | P | P | N |

A/A – Acid slant / Acid butt, G+ – Gas produced,
P – Positive, N – Negative
MR – Methyl Red, VP – Voges-Proskauer

**4. Antimicrobial sensitivity testing:**

Routine disk susceptibility testing of the Klebsiella isolates were performed by modified Kirby-Bauer Method in Muller Hinton agar medium obtained from Himedia. The results are affected

by the thickness of the medium and the inoculum density.[5] To overcome excessive thickness, in a plate of 90 mm diameter, 14 ml of medium was poured, to obtain a thickness of 4mm of media.

**Preparation of Inoculum (Growth Method):**[37]

1. The inoculum was prepared by touching with a wire loop the tops of four or five similar appearing, well isolated colonies on an agar plate culture, and inoculated onto 4 to 5 ml of nutrient broth.
2. The culture was incubated at 35˚ C until it matched in density with 0.5 McFarland's standard, which corresponds to 150 million organisms/ml.
3. Within fifteen minutes after adjusting the turbidity of the inoculum, a sterile nontoxic cotton swab was dipped into the inoculum suspension and the swab rotated several times with firm pressure on the inside wall of the tube to remove excess fluid.
4. The dried surface of a Mueller-Hinton agar plate that has been brought to room temperature is inoculated by streaking the swab three times over the entire agar surface, rotating the plate approximately 60˚ each time to ensure an even distribution of the inoculum. Finally, the rim of the agar was

swabbed, and the lid of the dish was replaced. Atleast 3 to 5 minutes was allowed but not longer than 15 minutes, for the surface of the agar to dry before placing the antibiotic discs.

**Testing of antibiotics:**

1. Appropriate antimicrobial-impregnated discs of 6mm diameter obtained from HiMedia were placed on the surface of the agar, using forceps.
2. Each disc was gently tamped down on to the agar to provide uniform contact. A disc once it has contacted the agar was not moved, because some of the drug diffuses almost immediately. Discs were evenly distributed on the agar so that they are not closer to each other.
3. The plates were inverted and placed in a 35˚ C air incubator for 16 – 18 hours

**Interpretation:**

**Measurement of zone diameters**: The plates after overnight incubation were examined. With the use of sliding calipers, a ruler or a template, the zones of complete growth inhibition around each of the discs were carefully measured to within the nearest millimeter. The diameter of the disc was included in this measurement.

All measurements were made with the unaided eye, while viewing the back of the Petri dish with reflected light against a black, non-reflecting back ground. The plates were viewed from a directly vertical line of sight to avoid any parallax that may result in misreading.

**General precautions:**

- Working stock of antibiotic discs were stored in sealed containers with a dessicant at less than 8˚ C, and brought to room temperature slowly to minimize condensation of moisture, which may lead to hydrolysis of the antibiotic.
- Care was taken not to leave the inoculated plates at room temperature before and after placing the discs.
- Plates were incubated not stacked more than three high.[41]

**5. Initial Screening test:** The sensitivity of the isolates to Third Generation Cephalosporins ceftazidime, and cefotaxime, each 30 µg/disc and to the other antibiotics such as ampicillin (10 µg), amoxicillin (10 µg), co-trimoxazole (1.25/23.75 µg), gentamicin (10 µg), amikacin (30 µg), Ciprofloxacin (5 µg) and imipenem (10 µg), (Hi-Media, India) was determined by the disc diffusion method.The results were interpreted as per Clinical Laboratory Standards Institute (CLSI)

recommendations.[17] Escherichia coli ATCC 25922 strain was used for quality control. Isolates with resistance or with decreased susceptibility (intermediate by CLSI criteria) to any of the Third Generation Cephalosporins were selected for further study.

| Sl. No. | Drug | Disk Content µg | Resistant mm or less | Intermediate mm | Sensitive mm or more |
|---|---|---|---|---|---|
| 1 | Ampicillin | 10 | 13 | 14-16 | 17 |
| 2 | Amoxycillin | 10 | 14 | 15-16 | 17 |
| 3 | Co-trimoxazole | 1.25+23.75 | 10 | 11-15 | 16 |
| 4 | Gentamycin | 10 | 12 | 13-14 | 15 |
| 5 | Amikacin | 30 | 14 | 15-16 | 17 |
| 6 | Ciprofloxacin | 5 | 15 | 16-20 | 21 |
| 7 | Cefotaxime | 30 | 14 | 15-22 | 23 |
| 8 | Ceftazidime | 30 | 14 | 15-17 | 18 |
| 9 | Imipenem | 10 | < 13 | 14-15 | > 16 |

**6. Phenotypic Confirmatory test**[17,39]**:** Antibiotic sensitivity testing was done on Mueller Hinton agar with 0.5 McFarland's standard of the organism[42]. The drugs used were Cefotaxime and Ceftazidime each 30 µg alone and in combination with Clavulanic acid 10 µg. Organisms with 5 mm increase in zone of inhibition with drug and Clavulanic acid, were confirmed as ESBLs.

**7. Double Disc Diffusion Synergy Test (DDST)[59]:** In the DDST, synergy was determined between a disc of augmentin (20 mg amoxycillin and 10 mg clavulanic acid) and a 30 mg disc of Third Generation Cephalosporin test antibiotic placed at a distance of 30 mm apart on a lawn culture of the resistant isolate under test on Mueller-Hinton Agar (MHA, Hi-Media). The test organism was considered to produce ESBL, if the zone size around the test antibiotic disc increased towards the augmentin disc. This increase occurs because the clavulanic acid present in the augmentin disc inactivates the ESBL produced by the test organism

**8. Minimum Inhibitory Concentration (MIC) determination:** Done by agar dilution method: Muller Hinton agar was prepared in tubes and autoclaved. It is then allowed to cool in a 50$^{o}$C water bath. Serial dilution of the 3 GCs ceftazidime and cefotaxime were prepared in sterile distilled water to give a final concentration ranging from 2µg – 2048µg/ml of agar.

After adding the drugs to the medium at 50$^{o}$ C it was mixed well and poured into sterile Petri dishes. (The media was used immediately otherwise potency of drugs will be affected. We can inoculate upto 12 different organisms in a single plate).

A control plate containing the test medium without the antibiotic was prepared for each series of test.

Plates of various concentrations were divided into required number (9-12 divisions / plate) 0.003 ml of inoculum was put into the appropriate quadrant and incubated at 37$^{o}$C for 16-20 hrs.

Minimum inhibitory concentration was the lowest concentration at which no visible growth occurs.

Isolates were tested for various concentrations of cephalosporin combined with 2µg/ml of clavulanic acid from 0.5µg to 2048µg / ml of agar and the MIC determined.

## 9. CLSI 2006 Protocol[16]:

According to CLSI guidelines 2006,

| | **Initial Screening Test** (In Mueller Hinton Agar) | **Phenotypic Confirmatory Test** (In Mueller Hinton Agar) |
|---|---|---|
| Antimicrobial Disc Concentration | Ceftazidime 30 µg<br>Cefotaxime 30 µg | Ceftazidime 30 µg and<br>Ceftazidime / Clavulanic acid 30/10 µg<br>&<br>Cefotaxime 30 µg and<br>Cefotaxime / Clavulanic acid 30/10 µg<br>(Confirmatory test requires all the four tests) |
| Results | Ceftazidime ≤22mm<br>Cefotaxime ≤27mm | ≥ 5mm increase in zone diameter with and without Clavulanic acid. |
| Quality Control | Klebsiella pneumoniae ATCC 700603<br>Escherichia coli ATCC 25922 | |
| Expected zone size | **Klebsiella pneumoniae ATCC 700603 –**<br>Ceftazidime : 10 – 18 mm<br>Cefotaxime : 17 – 25 mm<br>≥ 5mm increase in zone for Ceftazidime with and without Clavulanic acid<br>≥ 3mm increase in zone for Cefotaxime with and without Clavulanic acid<br>**Escherichia coli ATCC 25922 –**<br>≤ 2mm increase in zone with and without Clavulanic acid<br><br>Along with ESBL confirmatory tests, ATCC Klebsiella and ATCC Escherichia coli were done. | |

## 10. POLYMERASE CHAIN REACTION FOR DETECTION OF ESBL PRODUCING GENES

## (a) Identification of SHV, CTX-M-3 and CTX-M-14 GENES

**1. DNA Extraction**[45,46] was done by alcohol extraction method from young broth culture of the organisms**.**

**2 .Polymerase Chain Reaction**

PCR was carried out in a Peltier Thermal cycler (PTC 200) MJ Research PCR which had an initial denaturation for about 5 minutes at 94 $^{o}$C. Each cycle had a 2$^{nd}$ denaturation for about 1 minute.

Annealing at 58 $^{o}$ C was done for 1 minute.

Thirty five cycles were performed and a final extension was done for 10 minutes at 72 $^{o}$ C

**3. Gel Documentation**:

After the reaction, 25 µl. of the amplified samples were run on a 1.5 % agarose gel and electrophoresis done at 50 V with 1 x Tris Acetate EDTA buffer. Amplicons were visualized using Ethidium bromide staining and scored using 100 bp DNA ladder as reference.
Gels were viewed in a U - V gel documentation Unit and Photographed.

**4. Primer Sequences used:**

**CTX-M3F 5' AATCACTGCGCCAGTTCACGCT 3'**

| | |
|---|---|
| **CTX-M3R** | **5' GAACGTTTCGTCTCCCAGCTGT 3'** |
| **CTX-M14F** | **5' TACCGCAGATAATACGCAGGTG 3'** |
| **CTX-M14R** | **5' CAGCGTAGGTTCAGTGCGATCC 3'** |
| **SHV-F** | **5' AACGGAACTGAATGAGGCGCT 3'** |
| **SHV-R** | **5' TCCACCATCCACTGCAGCAGCT 3'** |

## RESULTS

Klebsiella isolates obtained from various clinical samples in the Department of Microbiology, Government Stanley Hospital were studied from June, 2006 to May, 2007 to find out the incidence of Extended Spectrum Beta Lactamases (ESBL) producers among them. Study included patients of both sexes and of all age groups. The Specimen included were Urine, Sputum, wound swab and Blood.

Out of the 204 Klebsiella isolates obtained, 111 were Klebsiella pneumoniae and 93 were Klebsiella oxytoca. 121 isolates out of 204 were positive for Extended Spectrum Beta Lactamase production and 83 were found to be negative for enzyme production.

The Results were analyzed as follows.

## Table 1: Total ESBL klebsiella isolates

| Number of Isolates | ESBL Klebsiella | Non-ESBL Klebsiella |
|---|---|---|
| 204 | 121 (59.31%) | 83 (40.69%) |

Total number of Klebsiella isolates taken for study were 204, of which 121 (59.31%) were positive for Extended spectrum Beta lactamase production and 83 (40.69%) isolates were found to be negative.

## Table 2: Age And Sex Distribution Of Klebsiella Isolates ( n = 204)

| Age | Male | Female | Total |
|---|---|---|---|
| < 20 | 22 | 43 | 65 |
| 20-30 | 13 | 13 | 26 |
| 31-40 | 17 | 7 | 24 |
| 41-50 | 14 | 28 | 42 |
| > 50 | 27 | 20 | 47 |

| | | | |
|---|---|---|---|
| TOTAL | 93 (45.59 %) | 111 (54.41 %) | 204 |

Total numbers of isolates taken for study were 204, which included total of 93 males (45.59 %) and 111 females (54.41 %). Among them, 22 males and 43 females were less than 20 years, between 20-30 years of age there were 13 males and 13 females, between 31-40 years 17 males and 7 females, between 41-50 years there were 14 males and 28 females and over 50 years there were 27 males and 20 females.

**Table 3: ESBL positive & negative Klebsiella in different specimen (n=204)**

| Specimen | Total | ESBL Positive | ESBL Negative |
|---|---|---|---|
| **Urine** | 97 | 64 (65.98%) | 33 (34.02%) |
| **Pus** | 51 | 27 (52.94%) | 24 (47.06%) |
| **Sputum** | 37 | 21 (56.76%) | 16 (43.24%) |
| **Blood** | 19 | 9 (47.37%) | 10 (52.63%) |
| **Total** | 204 | 121 (59.31%) | 83 (40.69%) |

Out of the 97 urinary Klebsiella isolates, 64 (65.98 %) were ESBL positive, and 33 (34.02 %) were ESBL negative. Of the 51 isolates from Pus specimen, 27 (52.94%) were ESBL Positive and 24 (47.06%) were ESBL Negative. Among the 37 Sputum isolates, 21 (56.76%) were ESBL Positive and 16 (43.24%) were ESBL Negative. And, of the 19 Blood isolates, 9 (47.37%) were ESBL Positive and 10 (52.63%) were ESBL Negative.

**Table 4: ESBL producers among Klebsiella species (n=121)**

| | ESBL Positive | Klebsiella pneumoniae | Klebsiella oxytoca |
|---|---|---|---|
| **Urine** | 64 | 16 (25 %) | 48 (75 %) |
| **Pus** | 27 | 18 ( 66.66%) | 9 ( 33.34%) |

| | | | |
|---|---|---|---|
| **Sputum** | 21 | 21 (100 %) | 0 |
| **Blood** | 9 | 9 (100 %) | 0 |
| **Total** | 121 | 64 ( 52.9%) | 57 (47.1 %) |

Out of the 64 urinary ESBL positive Klebsiella isolates, 16 (25 %) were Klebsiella pneumoniae, and 48 (75 %) were Klebsiella oxytoca. Of the 27 isolates from Pus specimen, 18 (66.66 %) were Klebsiella pneumoniae and 9 (33.34 %) were Klebsiella oxytoca. Among the 21 Sputum isolates, all 21 (100 %) were Klebsiella pneumoniae. And, of the 9 Blood isolates, all 9 (100 %) were Klebsiella pneumoniae.

Table 5: Number of Non-ESBL producing Klebsiella species (n=83)

| | **ESBL Negative** | **Klebsiella pneumoniae** | **Klebsiella oxytoca** |
|---|---|---|---|
| **Urine** | 33 | 4 (12.12 %) | 29 (87.88 %) |
| **Pus** | 24 | 19 (79.16 %) | 5 (20.84%) |
| **Sputum** | 16 | 14 (87.5 %) | 2 (12.5 %) |
| **Blood** | 10 | 10 (100 %) | 0 |
| Total | 83 | 47 | 36 |

Out of the 33 urinary ESBL positive Klebsiella isolates, 4 (12.12 %) were Klebsiella pneumoniae, and 29 (87.88 %) were Klebsiella oxytoca. Of the 24 isolates from Pus specimen, 19 (79.16 %) were Klebsiella pneumoniae and 5 (20.84 %) were Klebsiella oxytoca. Among the 16 Sputum isolates, 14 (87.5 %) were Klebsiella pneumoniae and 2 (12.5 %) were Klebsiella oxytoca. And, of the 10 Blood isolates, all 10 (100 %) were Klebsiella pneumoniae.

**Table 6: Sex-wise breakup of ESBL Klebsiella species (n=204)**

| | **ESBL** | **Non-ESBL** | Total |
|---|---|---|---|

| | | | |
|---|---|---|---|
| **Male** | 56 (60.22 %) | 37 (39.78 %) | 93 |
| **Female** | 65 (58.56 %) | 46 (41.44 %) | 111 |
| Total | 121 | 83 | 204 |

Out of the 93 Klebsiella isolates from male patients, 56 (60.22 %) were ESBL positive Klebsiella, and 37 (39.78 %) were ESBL negative. Out of the 111 Klebsiella isolates from female patients, 65 (58.56 %) were ESBL positive Klebsiella, and 46 (41.44 %) were ESBL negative.

**Table 7: Number of ESBL producing Klebsiella species in OP/IP (n=121)**

| | **ESBL** | **Non-ESBL** | Total |
|---|---|---|---|
| **OP** | 28 (70 %) | 12 (30 %) | 40 |
| **IP** | 93 (56.7 %) | 71 (43.3 %) | 164 |
| Total | 121 | 83 | 204 |

Out of the 40 Klebsiella isolates from Outpatient department, 28 (70 %) were ESBL positive Klebsiella, and 12 (30 %) were ESBL negative. Out of the 164 Klebsiella isolates from Inpatients, 93 (56.7 %) were ESBL positive Klebsiella, and 71 (43.3 %) were ESBL negative.

## Table 8: ESBL positive Klebsiella species by different methods (n=121)

| Methods | DDST | MIC | Meropenem sensitivity |
|---|---|---|---|
| Positive Isolates | 121 (100%) | 121 (100%) | 121 (100%) |

All 121 Klebsiella species found ESBL positive by screening method were subjected to further tests, and by all methods they were confirmed as ESBL positive.

## Table 9: Antibiotic Sensitivity pattern of ESBL producing Klebsiella (n=121)

| ANTIBIOTIC | Sensitive ESBL Klebsiella |
|---|---|
| *Ampicillin* | 0 |
| **Amoxicillin** | 0 |
| **Cotrimoxazole** | 0 |
| **Gentamycin** | 9 (7.44 %) |
| **Amikacin** | 66 (54.54 %) |
| **Ciprofloxacin** | 20 (16.53 %) |
| **Cefotaxime** | 0 |
| **Ceftazidime** | 6 (4.96 %) |
| **Imipenem** | 121 (100 %) |

Out of the 121 ESBL positive Klebsiella isolates, none were sensitive to Cotrimoxazole, 9 (7.44 %) were sensitive to Gentamycin, 66 (54.54 %) were sensitive to Amikacin, 20 (16.53 %) were sensitive to Ciprofloxacin and all were sensitive to Imipenem.

**Table 10: Antibiotic Sensitivity of ESBL Klebsiella pneumoniae (n=64)**

| ANTIBIOTIC | **Klebsiella pneumoniae** |
|---|---|
| ***Ampicillin*** | 0 |
| Amoxicillin | 0 |
| Cotrimoxazole | 0 |
| Gentamycin | 3(4.69 %) |
| Amikacin | 33(51.56 %) |

| | |
|---|---|
| Ciprofloxacin | 12(18.75 %) |
| Cefotaxime | 0 |
| Ceftazidime | 5(7.81%) |
| Imipenem | 64(100 %) |

Of 64 ESBL positive Klebsiella pneumoniae, none were sensitive to Cotrimoxazole, 3 (4.69 %) were sensitive to Gentamycin, 33 (51.56 %) to Amikacin, 12 (18.75 %) to Ciprofloxacin and all were sensitive to Imipenem.

**Table 11: Antibiotic Sensitivity of ESBL producing Klebsiella oxytoca (n=57)**

| ESBL | Klebsiella oxytoca |
|---|---|
| ***Ampicillin*** | 0 |
| Amoxicillin | 0 |
| Cotrimoxazole | 0 |
| Gentamycin | 6(10.53 %) |
| Amikacin | 33(57.89 %) |
| Ciprofloxacin | 8(14.04) |
| Cefotaxime | 0 |
| Ceftazidime | 1(1.75 %) |
| Imipenem | 57(100 %) |

Out of the 57 ESBL positive Klebsiella oxytoca, none were sensitive to Cotrimoxazole, 6 (10.53 %) were sensitive to Gentamycin, 33 (57.89 %) were sensitive to Amikacin, 8 (14.04 %) were sensitive to Ciprofloxacin and all were sensitive to Imipenem.

## Table 12: Antibiotic Sensitivity pattern of ESBL negative Klebsiella (n = 83)

| ANTIBIOTIC | Non-ESBL Klebsiella |
|---|---|
| ***Ampicillin*** | 31 (37.35 %) |
| Amoxicillin | 28 (33.73 %) |
| Cotrimoxazole | 39 (46.99 %) |
| Gentamycin | 68 (81.93 %) |
| Amikacin | 83 (100 %) |
| Ciprofloxacin | 78 (93.98 %) |
| Cefotaxime | 83 (100 %) |
| Ceftazidime | 83 (100 %) |
| Imipenem | 83 (100 %) |

Out of the 83 ESBL negative Klebsiella isolates, 31 (37.35 %) were sensitive to ampicillin, 28 (33.73 %) were sensitive to amoxicillin, 39 (46.99 %) were sensitive to Cotrimoxazole, 68 (81.93 %) were sensitive to Gentamycin, 78 (93.98 %) were sensitive to Ciprofloxacin and all were sensitive to amikacin, Cefotaxime, Ceftazidime and Imipenem.

## Table 13: Antibiotic Sensitivity of ESBL negative Klebsiella pneumoniae (n=47)

| ANTIBIOTIC | Klebsiella pneumoniae |
|---|---|
| ***Ampicillin*** | 21 (44.68%) |
| Amoxicillin | 18 (38.30%) |
| Cotrimoxazole | 18 (38.30%) |
| Gentamycin | 40 (85.11%) |
| Amikacin | 47 (100%) |
| Ciprofloxacin | 45 (95.74%) |
| Cefotaxime | 47 (100%) |
| Ceftazidime | 47 (100%) |
| Imipenem | 47 (100%) |

Out of the 47 ESBL negative Klebsiella pneumaoniae, 21 (44.68 %) were sensitive to ampicillin, 18 (38.30 %) were sensitive to amoxicillin and Cotrimoxazole, 40 (85.11 %) were sensitive to Gentamycin, 45 (95.74 %) were sensitive to Ciprofloxacin and all were sensitive to amikacin, Cefotaxime, Ceftazidime and Imipenem.

**Table 14: Antibiotic Sensitivity of ESBL negative Klebsiella oxytoca (n=36)**

| ESBL | Klebsiella oxytoca |
|---|---|
| ***Ampicillin*** | 10 (27.78 %) |
| Amoxicillin | 10 (27.78 %) |
| Cotrimoxazole | 21 (58.33 %) |
| Gentamycin | 28 (77.78 %) |
| Amikacin | 36 (100 %) |
| Ciprofloxacin | 33 (91.67 %) |
| Cefotaxime | 36 (100 %) |
| Ceftazidime | 36 (100 %) |
| Imipenem | 36 (100 %) |

Out of the 36 ESBL negative Klebsiella oxytoca, 10 (27.78 %) were sensitive to ampicillin and amoxicillin, 21 (58.33 %) were sensitive to Cotrimoxazole, 28 (77.78 %) were sensitive to Gentamycin, 33 (91.67 %) were sensitive to Ciprofloxacin and all were sensitive to amikacin, Cefotaxime, Ceftazidime and Imipenem.

## Table 15: Minimum Inhibitory Concentration (MIC) of ESBL Klebsiella to Cefotaxime (n=121)

| | Concentration of Cefotaxime in Agar (µg/ml) | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Total | 2 | 4 | 8 | 16 | 32 | 64 | 128 | 256 | 512 | 1024 | 2048 |
| **Klebsiella pneumoniae** | 64 | 0 | 0 | 0 | 0 | 0 | 3 | 21 | 32 | 46 | 60 | 64 |
| **Klebsiella oxytoca** | 57 | 0 | 0 | 0 | 0 | 0 | 0 | 19 | 26 | 35 | 51 | 57 |

Minimum inhibitory concentration of Cefotaxime for the ESBL producing organisms in the study was between 64 µg/ml of agar to 2048 µg/ml of agar.

## Table 16: Minimum Inhibitory Concentration (MIC) of ESBL Klebsiella to Cefotaxime with 2µg/ml Clavulanate (n=121)

| | Concentration of Cefotaxime with 2µg/mlClavulanic acid in Agar (µg/ml) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Total | 0.5 | 1 | 2 | 4 | 8 | 16 | 32 | 64 | 128 |
| **Klebsiella pneumoniae** | 64 | 3 | 27 | 43 | 48 | 52 | 60 | 63 | 64 | 64 |
| **Klebsiella oxytoca** | 57 | 2 | 18 | 39 | 45 | 48 | 51 | 53 | 57 | 57 |

Minimum inhibitory concentration of Cefotaxime for the ESBL producing organisms in the study was between 0.5 µg/ml of agar to 64 µg/ml of agar in the presence of clavulanic acid at a concentration of 2 µg/ml of agar showing 8 fold reductions in MIC.

## Table 17: Minimum Inhibitory Concentration (MIC) of ESBL Klebsiella to Ceftazidime (n=121)

| | Concentration of Ceftazidime in Agar (µg/ml) | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Total | 2 | 4 | 8 | 16 | 32 | 64 | 128 | 256 | 512 | 1024 | 2048 |
| **Klebsiella pneumoniae** | 64 | 0 | 0 | 0 | 0 | 0 | 5 | 32 | 43 | 57 | 62 | 64 |
| **Klebsiella oxytoca** | 57 | 0 | 0 | 0 | 0 | 0 | 3 | 27 | 38 | 51 | 55 | 57 |

Minimum inhibitory concentration of Ceftazidime for the ESBL producing organisms in the study was between 64 µg/ml of agar to 2048 µg/ml of agar

## Table 18: Minimum Inhibitory Concentration (MIC) of ESBL Klebsiella to Ceftazidime with 2µg/ml Clavulanate (n=121)

| | Concentration of Ceftazidime with 2µg/mlClavulanic acid in Agar (µg/ml) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Total | 0.5 | 1 | 2 | 4 | 8 | 16 | 32 | 64 | 128 |
| Klebsiella pneumoniae | 64 | 23 | 57 | 60 | 64 | 64 | 64 | 64 | 64 | 64 |
| Klebsiella oxytoca | 57 | 17 | 56 | 56 | 57 | 57 | 57 | 57 | 57 | 57 |

Minimum inhibitory concentration of Ceftazidime for the ESBL producing organisms in the study was between 0.5 µg/ml of agar to 4 µg/ml of agar in the presence of clavulanic acid at a concentration of 2 µg/ml of agar showing 8 fold reductions in MIC.

## Table 19: PCR Sequencing of ESBL positive Klebsiella (n=30)

| | Total | SHV | CTX-M3 | CTX-M14 |
|---|---|---|---|---|
| Klebsiella pneumoniae | 15 | 12 | 7 | 3 |
| Klebsiella oxytoca | 15 | 11 | 5 | 0 |

**PCR for determination of SHV, CTX-M-3, and CTX-M-14 was done. SHV gene in 12 Klebsiella pneumoniae and 11 Klebsiella oxytoca isolates, CTX-M-3 was found in 7 Klebsiella pneumoniae and 5 Klebsiella oxytoca isolates. CTX-M-14 was found in 3 Klebsiella pneumoniae and none in any of the Klebsiella oxytoca isolates. 7 Klebsiella pneumoniae and 4 Klebsiella oxytoca isolates showed both SHV and CTX-M-3.**

## Primer Sequences:

CTX-M3F 5' AATCACTGCGCCAGTTCACGCT 3'

CTX-M3R 5' GAACGTTTCGTCTCCCAGCTGT 3'

CTX-M14F 5' TACCGCAGATAATACGCAGGTG 3'

CTX-M14R 5' CAGCGTAGGTTCAGTGCGATCC 3'

SHV-F 5' AACGGAACTGAATGAGGCGCT 3'

SHV-R 5' TCCACCATCCACTGCAGCAGCT 3'

## DISCUSSION

The wide spread use of antibiotics in hospitals has led to emergence of multidrug resistant organisms of low virulence like Klebsiella causing serious opportunistic infections. Over the last 15 years numerous outbreaks of infection with organisms producing extended spectrum β-lactamases (ESBLs) have been observed world wide.

The advent of ESBL producers has posed a great threat to the use of many classes of antibiotics particularly cephalosporins.

There are indications that poor outcome occurs when patients with serious infections due to ESBL producing organisms are treated with antibiotics to which the organism is resistant.

ESBL producing Klebsiella pneumoniae were first reported in 1983 from Germany and since then a steady increase in resistance against cephalosporins has been seen. ESBLs are encoded by transferable conjugative plasmids, which also quite often code resistant determinants to other antibiotics. An ESBL variant may be selected de novo in a given hospital or it may be introduced from another centre. Its further spread within the hospital can be consequence of plasmid transmission. Persistence and outbreaks of ESBL producers have been convincingly correlated with extensive use of cephalosporins.

Klebsiella pneumoniae is an important cause of nosocomial infection and infections due to ESBL producers are of concern as third generation cephalosporins (3GC) are commonly used for treatment of infections due to gram-negative organisms. These infections are difficult to control as they are usually associated with resistance to aminoglycosides.

The clinical microbiology laboratory has the task of screening and confirming isolates for ESBL production. This is a challenge for the laboratory to detect ESBL-containing gram negative bacilli

because they can appear susceptible in vitro to certain beta-lactam antimicrobial agents yet result in clinical treatment failure.

The prevalence of ESBLs among Clinical Isolates varies greatly worldwide and in geographic areas and is rapidly changing over time.

In the present study, the occurrence of ESBL producing Klebsiella species were 121 (59.31 %), correlating with Indian studies like 56 % obtained by Amita Jain et al[3], 58 % by Bithika Duttaroy et al[12], 58 % by Gunseren et al[29] and 53 % by Revathi et al[53]. Values higher than our study were obtained in studies like the 72% positive by Shukla et al[54], 73% positive by Singhal et al[55], 80 % positive reported by Purva et al[52], 86 % reported by Prabha et al[51] and 87 % reported by Subha et al[5] .

But, the percentage of ESBLs were significantly higher than European and US studies which gave 21 % Fluit et al[21], Albertini et al[2], 23 % Meifang et al[44] and 29 % Jacoby et al[24], and Indian studies like 8.5% by Rodrigues et al[14], 21% by Menon et al[45] and 14% by Kumar et al[38].

The Male: Female ratio of Klebsiella samples analyzed was 93:111 (1:1.2).

Of the 97 urinary isolates of Klebsiella, 64 (65.98 %) were ESBL positive and from 51 Pus specimen 27 (52.94 %) were

ESBL positive. Likewise, 21 (56.76 %) from 37 Sputum specimen and 9 (47.37 %) from blood specimen were ESBL positive.

In the study by Bithika Duttaroy et al[12], 36 % in Urine specimen, 27.7 % in Pus, 18.2 % in Blood and 15.1 % in others were ESBL positive.

In a study by Purva et al[52], 58 % in Urine specimen, 52 % in Pus, 87 % in Blood and 74 % in others were ESBL positive

In the present study, among the 121 ESBL positive Klebsiella, 64 (52.9 %) of Klebsiella pneumoniae and 57 (47.1 %) of Klebsiella oxytoca were obtained.

In a study by Maurizio et al[43], ESBL positive Klebsiella pneumoniae obtained were 74.7 % and ESBL positive Klebsiella oxytoca were 77.78 %. Coudron et al[49] obtained 37 % ESBL Klebsiella pneumoniae and 4 % ESBL positive Klebsiella oxytoca. As per the study of Luzzaro et al[22], 20 % Klebsiella pneumoniae isolates and 15.1 % of Klebsiella oxytoca were ESBL positive

In present study, out of the 64 urinary ESBL positive Klebsiella isolates, 16 (25 %) were Klebsiella pneumoniae, and 48 (75 %) were Klebsiella oxytoca. In a study by Bithika et al[12], of the total Klebsiella isolated from urine, 36 % were ESBL positive.

Of the 27 isolates from Pus specimen, 18 (66.66 %) were Klebsiella pneumoniae and 9 (33.34 %) were Klebsiella oxytoca. In a study by Kanungo et al[7], 43.75 % and 27.7 % by Bithika et al[12] of ESBL positive Klebsiella pneumoniae were obtained.

Among the 21 Sputum isolates, all 21 (100 %) were Klebsiella pneumoniae. In a study by Bithika et al[12], 15.1 % Klebsiella pneumoniae isolates were ESBL positive.

And, of the 9 Blood isolates, all 9 (100 %) were Klebsiella pneumoniae. In a study by Bithika et al[12], 18.2 % Klebsiella pneumoniae isolates were ESBL positive.

In our study, out of the 121 ESBL positive Klebsiella isolates, none were sensitive to Cotrimoxazole, 9 (7.44 %) were sensitive to Gentamycin, 66 (54.54 %) were sensitive to Amikacin, 20 (16.53 %) were sensitive to

Ciprofloxacin and all were sensitive to Imipenem, correlating with Hansotia et al[30].

And among the 83 ESBL negative Klebsiella isolates, 31 (37.35 %) were sensitive to ampicillin, 28 (33.73 %) were sensitive to amoxicillin, 39 (46.99 %) were sensitive to Cotrimoxazole, 68 (81.93 %) were sensitive to Gentamycin, 78 (93.98 %) were sensitive to Ciprofloxacin and all were sensitive to amikacin, Cefotaxime, Ceftazidime and Imipenem.

In a study by Baby Padmini et al[8], among ESBL producers, 86% were susceptible to amikacin, 25 % to gentamicin, 26 % to cotrimoxazole and 9 % to ciprofloxacin. And, among non-ESBL producers, 94 % were susceptible to amikacin, 92 % to gentamicin, 47 % to cotrimoxazole and 64 % to ciprofloxacin.

According to Amita et al[3], among ESBL producers, 41.4 % were susceptible to amikacin, 29.3 % to gentamicin, 31 % to cotrimoxazole and 32.8 % to ciprofloxacin. And, among non-ESBL producers, 81 % were susceptible to amikacin, 47.6 % to gentamicin, 38 % to cotrimoxazole and 81 % to ciprofloxacin.

Luzzaro et al[22] study gives a susceptibility of 51.9 % for amikacin, 43.2 % for Gentamicin and 56.8 % for Ciprofloxacin, in ESBL Klebsiella pneumoniae, and a susceptibility of 100 % for

amikacin, 72.2 % for Gentamicin and 72.2 % for Ciprofloxacin, in ESBL Klebsiella oxytoca.

In our study, out of the 121 ESBL positive Klebsiella isolates, (16.53 %) were sensitive to Ciprofloxacin, and among the 83 ESBL negative Klebsiella isolates, 78 (93.98 %) were sensitive to Ciprofloxacin. This correlates well with the study of Patterson et al[19], giving a 60 % ESBL positives in Ciprofloxacin resistant Klebsiella pneumoniae and 16 % in Ciprofloxacin sensitive isolates, thereby, concluding a positive association between Ciprofloxacin resistance and ESBL production.

By agar dilution method, Minimum inhibitory concentration for Cefotaxime for the 121 ESBL producing organisms varied from 64 µg/ml to 2048 µg/ml. This showed MIC 50s at 256 µg/ml and MIC 90s at 1024 µg/ml for Klebsiella species.

In combined with 2 µg/ml. of clavulanic acid, Cefotaxime gave a MIC reduced to 0.5 µg/ml - 8 µg/ml.

MIC for ceftazidime was between 64 µg/ml - 2048 µg/ml, with MIC 50s, 512 µg/ml.

In combination with 2 µg/ml of clavulanic acid, Ceftazidime MIC was reduced to 0.5 µg/ml - 8 µg/ml,. From a study by Bithika et al[12], MIC for third generation cephalosporins was between 2-1024 µg/ml and it was reduced to 0.25-128 µg/ml when clavulanic acid was added at a concentration of 2 µg/ml. So the present study correlates well with the study of Bithika et al[12].and Livermore et al[40].

Polymerase Chain Reaction for identification of SHV, CTX – M-3 and CTX-M-14 showed presence of SHV genes in 12 of Klebsiella pneumoniae and 11 Klebsiella oxytoca isolates, CTX-M-3 was identified in 7 Klebsiella pneumoniae and 5 Klebsiella oxytoca isolate. CTX-M-14 was identified in 3 Klebsiella pneumoniae isolates.

This correlates with the study of Ju-Hsin Chia et al[34] from Taiwan, where most of the Klebsiella pneumoniae isolates showed SHV. SHV was common in studies by Luzzaro et al[22], Shanmuganathan et al[56], Paterson et al[20], Miranda et al[28] and by Zoltan Pragai[62]. But, in a study by Chanawong et al[6], CTX was found to be common.

## SUMMARY

Two hundred and four Klebsiella isolates from varied specimen provided by patients attending Government Stanley Hospital, Chennai, between June, 2006 to May, 2007 formed the study group.

Specimens like urine, blood, wound swabs, sputum and blood were cultured and organisms identified by Gram's staining and various biochemical reactions, from a total of 121 isolates, as 64 (52.9 %) of Klebsiella pneumoniae, and 57 (47.1 %) of Klebsiella oxytoca.

Antibiotic susceptibility testing was done including third generation cephalosporins and Klebsiella species found to be resistant to third generation cephalosporin were screened for ESBL production by phenotypic Confirmatory method, the Double disc synergy test (DDST) and by Minimum Inhibitory Concentration technique to be 121 of 204 specimens.

ESBL Klebsiella pneumoniae among urine (66 %), pus (53 %), sputum (56.7 %) and blood (47.4 %).

MIC for the isolates was between 64- 2048 µg/ml of agar for cefotaxime and ceftazidime alone, which was reduced to 0.5 - 64 µg/ml and 0.5- 4 µg/ml of agar respectively in the presence of 2 µg/ml of agar.

All the 121 isolates (100%) were found to be sensitive to imipenem, and 7.44 % to Gentamicin, 54.54 % to Amikacin and

16.53 % to Ciprofloxacin, which could be economical alternatives to expensive drugs like Imipenem.

SHV gene, CTX-M-3 and CTX -M-14 were identified in the Klebsiella isolates.

## CONCLUSION

Our study gives 59.3 %, which is intermediate to the varied studies in India, but high compared to western countries like in US 4.2-44.0 per cent, in Canada 15-17 %, and in China 51 %[48]. In India, high prevalence of ESBL producing *Klebsiella* strains has been reported by various groups. Reported frequency of ESBL producing *Klebsiella* spp. from India ranged between 6 and 87 per cent[3].

The high percentage of ESBL producing *Klebsiella* spp may be due to the selective pressure imposed by extensive use of antimicrobials. The infection control implications of ESBL producing *Klebsiella* spp. are under-recognized.

Our results showed a high incidence of ESBL producing *Klebsiella* species in both In-patient and Outpatient. Routine detection of ESBL producing microorganisms is required by reliable laboratory methods and since most of these are multidrug

resistant, the therapeutic strategies to control infections in the hospital setup have to be carefully formulated.

Phenotypic confirmatory method is less worktime and less skill demanding while being economical, and will be the ideal method for confirmation of ESBL producers.

A fall in the incidence of ESBL producers, have been noted in France in a study after implementing an effective strategy against ESBLs were brought in. Such a workup is imminent for our country in the present scenario.

While ESBL producing organisms were 100% sensitive to Imipenem, they were also sensitive to cheaper drugs like, Gentamicin (7.44 %), Amikacin (54.54 %) and Ciprofloxacin (16.53 %). Hence if we screen carefully we can minimize the cost of treatment for the patients with infections due to ESBL producing organisms.

## APPENDIX

### 1. Brain Heart Infusion broth:-

| | Gm/Liter |
|---|---|
| Calf brain infusion from | 200 |
| Beef infusion from | 250 |
| Proteose peptone | 10 |
| Dextrose | 2 |
| Sodium chloride | 5 |
| Disodium phosphate | 2.50 |

Add the contents and dissolve by heating.

Adjust the pH to 7.4 + 0.2

Autoclave at 121$^{o}$C for 15 minutes.

## 2. Peptone Water:-

| | |
|---|---|
| Peptone | 10 g |
| Sodium chloride, NaCl | 5 g |
| Water | 1 litre |

Dissolve the ingredients in warm water, adjust the pH to 7.4-7.5 and filter. Distribute as required and autoclave at 121$^{o}$C for 15 min.

## 3. MacConkey agar:-

This is a useful medium for the cultivation of enterobacteria. It contains a bile salt to inhibit non-intestinal bacteria and lactose with neutral red to distinguish the lactose-fermenting coli forms from the lactose –non-fermenting salmonella and shigella groups. The concentration of sodium taurocholate may be reduced to suit less tolerant organisms. The omission of sodium chloride from the medium prevents the spreading of Proteus colonies.

| | |
|---|---|
| Peptone | 20 g |
| Sodium taurocholate, commercial | 5 g |
| Water | 1 litre |
| Agar | 20 g |
| Neutral red solution, 2% in 50% ethanol | 3.5 ml |

| | |
|---|---|
| Lactose, 10% aqueous solution | 100 ml |

Dissolve the peptone and taurocholate (bile salt) in the water by heating. Add the agar and dissolve it in the steamer or autoclave. If necessary, clear by filtration. Adjust the pH to 7.5. Add the lactose and the neutral red, which should be well shaken before use, and mix. Heat in the autoclave with 'free steam' (c. 100° C) for 1hr., then at 115° C for 15 min. Pour plates.

**4. Nutrient agar:-**

| | Gm/L |
|---|---|
| Peptic digest of animal tissue | 5.00 |
| Beef extract | 1.50 |
| Yeast extract | 5.00 |
| Agar | 15.00 |

Dissolve the contents in water and mix by heating Autoclave at 121° C for 15 minutes. Adjust pH to 7.4 + 0.2. Pour 20-25 ml of 9 cm dia. Petridishes to give 4 mm thickness.

**5. Blood agar:-**

| | |
|---|---|
| Sterile sheep blood | 50 ml |
| Peptone | 10 g |

| | |
|---|---|
| Beef extract | 3g |
| Sodium chloride | 5 g |
| Distilled water | 1000 ml |

Autoclave the nutrient agar base at 121° C for 15 minutes and blood with sterile precautions and distribute in Petri dishes.

**6. Muller Hinton agar:-**

| | | |
|---|---|---|
| Beef infusion | 300 | ml |
| Casein Hydrolysate | 17 | gm |
| Starch | 1.5 | gm |
| Agar | 10 | gm |
| Distilled water | 1000 | ml |

Emulsify the starch in a small amount of cold water, pour into the beef infusion and add the casein-Hydrolysate and the agar. Make up the volume to 1000 ml (1 litre) with distilled water. Dissolve the constituents by heating gently at 100° C with agitation.

Adjust the pH to 7.4. Dispense in screw-capped bottles and sterilize by autoclaving at 121 $^{o}$ C for 20 minutes. 20 to 25 ml of it is poured into petridishes of 9 cm diameter to give a thickness of 4mm.

## 7. McFarland's Turbidity Standard for inoculum preparation

A Barium sulphate 0.5 McFarland standards was prepared as follows

1. A 0.5 ml of 0.048mol/L of Barium chloride was added to 99.5 ml of 0.18 mol/L of $H_2SO_4$ with constant stirring to maintain a suspension.
2. Correct density of the turbidity standard was verified by using a spectrophotometer. The absorbance of 625nm should be 0.08 to0.10 for the 0.5 McFarland standards.
3. The Barium sulphate suspension was transferred in 4-6 ml to a screw capped tube of the same size as those used in growing or diluting the bacterial inoculam.
4. These tubes were tightly sealed and stored in the dark at room temperature.
5. The Barium sulphate turbidity standard was vigorously agitated before each use and inspected for a uniform turbid appearance.

# 8. DNA Extraction

Materials required

i) Overnight broth culture of the organism

ii) Lysozyme solution

iii) Proteinase K

iv) 10% SDS

v) 70% ETHANOL

vi) 95% ETHANOL

vii) Tris EDTA Buffer

viii) Sterile eppendorfs & Pipettes

ix) 3 M sodium acetate

x) Phenol: Chloroform 1:1

**Procedure**

1.5 ml of overnight broth culture was taken in an eppendorf tube and centrifuged at 10000 rpm for 4 minutes at 4 $^{o}$ C

Supernatant was discarded and pellet was dissolved in 467 ml. of Tris EDTA buffer. One Ml. of Lysozyme was added to the above solution and incubated for 1 hour at 30 $^{o}$ C

30 ml. of 10% SDS and 3 ml. of 20 mg./ml Proteinase Kinase K were mixed to the sample and incubated for 1 hour at 37 $^{o}$ C

Equal volume of phenol and chloroform (1:1) mixture was added and mixed until the phases were completely mixed.

Phase lock tubes were centrifuged at 12,000 rpm for 8 minutes to pellet the column

The solution was pipetted into phase lock tubes and centrifuged at 10000 rpm to purify the DNA. The clear upper layer was pipetted into a sterile eppendorf tube. Equal volume of chloroform was added and centrifuged at 10,000 rpm for 10 minutes.

The upper aqueous phase was again transferred to a new table and to it 1/10$^{th}$ volume of 3 M sodium acetate solution and 2 volumes of ice cold absolute alcohol were added and the DNA was precipitated. The tubes were again centrifuged at 10,000 rpm for 15 minutes and the supernatant was discarded.

Pellet was washed with 70% ethanol by spinning for 5 minutes at 10,000 rpm. 70% ethanol was discarded on the tissue paper and pellet was air dried completely. Extracted DNA was suspended in Tris EDTA buffer.

## 9. PCR Master Mix

| | |
|---|---|
| Milli Q water | 12 ml |
| 10 x buffer | 2.5 ml (1x) |
| 2 mm d NTPs | 2.5 ml. (0.2 m) |
| DNA (50 mg/ml) | 1 ml. |
| Primer F | 2.5 ml |
| R | 2.5 ml |
| Taq polymerase ( 2U/ml) | 2.0 ml. |

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